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Remarks
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(54) **Family of xanthan-based polysaccharide polymers including non-acetylated and/or non-pyruvylated gum**

(57) Variant xanthan gums including non-acetylated non-pyruvylated non-acetylated and non-pyruvylated. and fully-acetylated xanthan gums. In addition. in vitro and in vivo methods for the synthesis of these gums are disclosed Mutant **Xanthomonas campestris** strains useful in these synthesis are also specified

FIG. I.

EP 0 765 939 A2
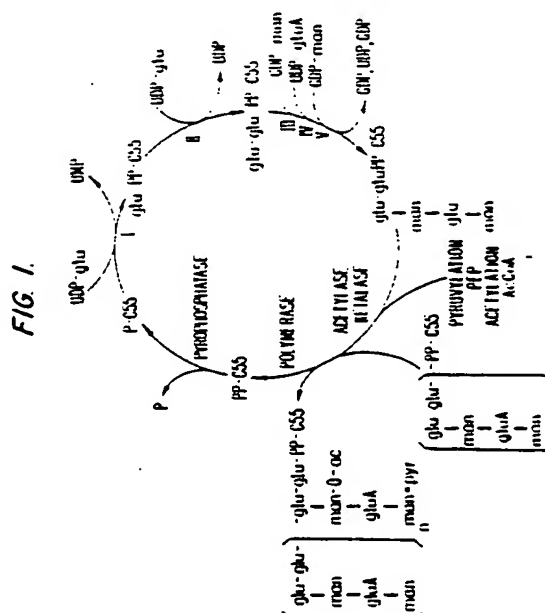
Description

## BACKGROUND OF THE INVENTION

This invention relates to polysaccharide polymers In particular it relates to xanthan-based polysaccharide polymers defined here as polymers structurally similar to xanthan gum and produced by components of the xanthan biosynthetic pathway including non-acetylated. non-pyruvylated non-acetylated and non-pyruvylated low pyruvylated and fully pyruvylated xanthan (pentamer) gums.

Xanthan gum is produced by bacteria of the genus <u>Xanthomonas</u> in particular by microorganisms of the species <u>X. campestris</u>. Xanthan gum is a widely used product due to its unusual physical properties. i.e its extremely high specific viscosity and its pseudoplasticity It is commonly used in foods as a thickening agent and in secondary or tertiary oil recovery as a mobility control and profile modification agent as well as in petroleum drilling fluids

Chemically. xanthan gum is an anionic heteropolysaccharide The repeating unit of the polymer is a pentamer composed of five sugar moieties. specifically two glucose one glucuronic acid and two mannose moieties These sugar residues are arranged such that the glucose moieties form the backbone of the polymer chain with side chains of mannose-glucuronic acid-mannose residues generally extending from alternate glucose moieties Usually. this basic structure is specifically acetylated and pyruvylated. as described for example. by Janson P E Kenne L and Lindberg. B in Carbohydrate Research. <u>45</u> 275-292 (1975) and Melton L D Minot L . Rees. D A and Sanderson. G R.. in Carbohydrate Research <u>46</u> 245-257 (1976) each of which is specifically incorporated herein by reference The extent of acetylation and pyruvylation is known to vary. The structure of xanthan gum is depicted below

Xanthan
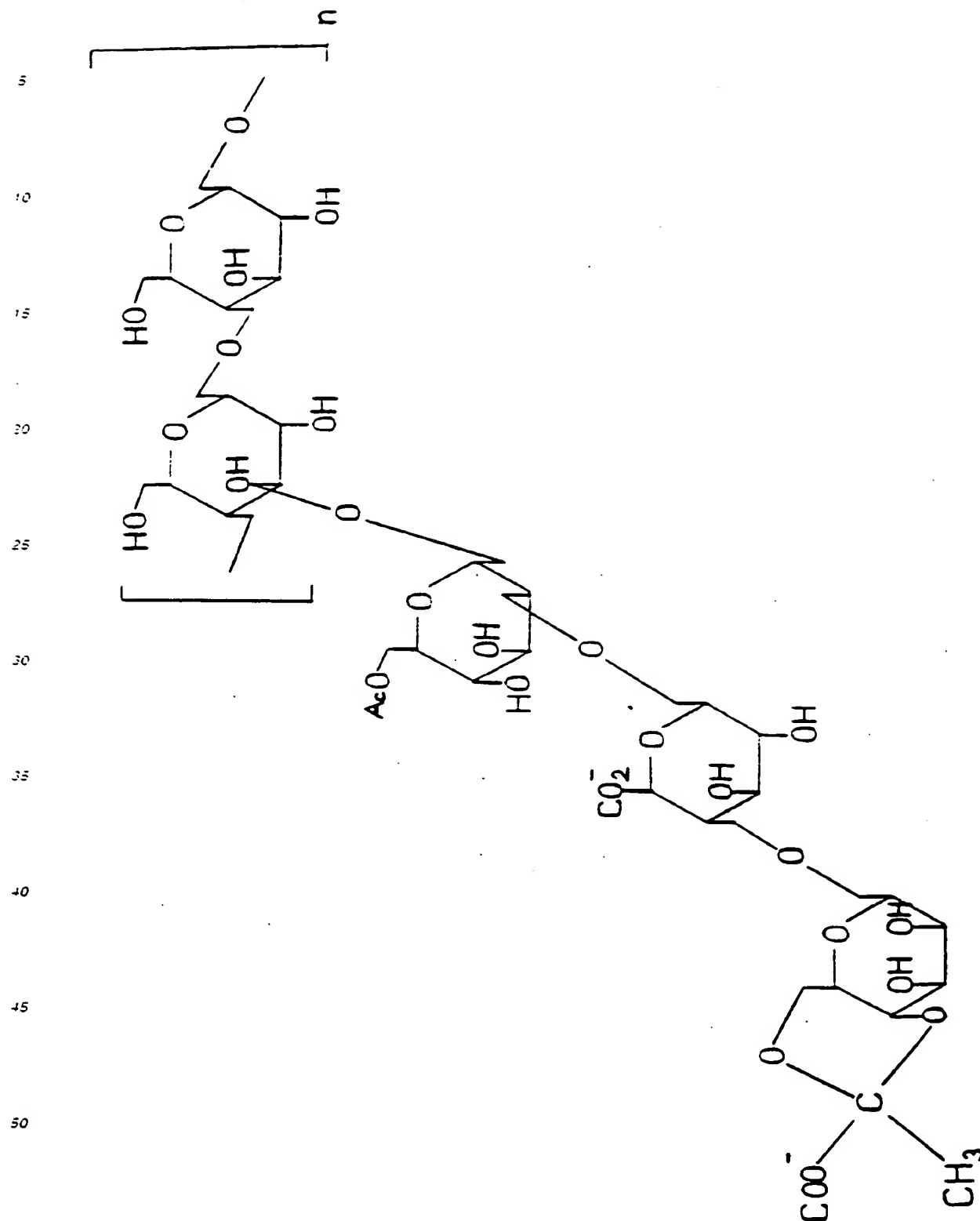
In spite of the broad utility of naturally-occurring xanthan gum. there are some situations where its physical properties become limiting. In particular. in secondary or tertiary oil recovery it is not uncommon for the temperature of the oil bearing reservoir and the salt concentrations in the reservoir brine to be higher than are optimal for xanthan solutions.

When these conditions occur xanthan can precipitate flocculate and/or lose its viscosity Therefore new viscosifying products which perform well at various conditions encountered during oil recovery such as high temperature and high salt concentrations would be desirable

The present invention discloses a family of xanthan-based polysaccharides having improved properties relative to naturally-occurring xanthan gum Modifications of xanthan gum have been previously described For example Bradshaw et al (Carbohydrate Polymers 3 23-38 (1983)) describe methods for preparing chemically-modified xanthan gum which are deacetylated or depyruvylated Various means of chemically deacetylating xanthan gum produced by Xanthomonas campestris also are described in U S Patent Nos 3 000 790 and 3 054 689 To date the sole method utilized for these deacetylation processes has been through chemical removal of the acetate moieties from normally acetylated xanthan gum It has been found that chemical processes for deacetylating xanthan gums result in a number of undesirable side effects and may cause hydrolysis of the glycosidic backbone resulting in an irreversible change in the conformation of the molecule and lowered molecular weight

Some of the rheological properties of deacetylated xanthan in aqueous media are known. See e g . Tako and Nakamura Agric Biol Chem 48 2987-2993 (1984) and U S Patents Nos 3 000 790 and 3 054 689 Also. a method of increasing the viscosity of an aqueous solution using a deacetylated poly-saccharide is described in U S Patent No 3.096 293 Thus a method for obtaining non-acetylated xanthan which does not cause untoward side effects has been sought.

Xanthan gum can be chemically depyruvylated as well as described by Holzwarth and Ogletree in Carbo Res 76 277-280 (1979) This chemical method of depyruvylation also can alter the xanthan polymeric unit and/or cause hydrolysis of the glycosidic backbone While a strain of X campestris has been described in U S Patent No 4 296 203 which produces non-pyruvylated xanthan gum. this non-pyruvylated gum was either fully acetylated or deactylated using chemical means The present inventors believe that a xanthan polysaccharide that is both non-acetylated and non-pyruvylated will have improved rheological and viscosifying properties and have therefore sought such a gum along with a microbial method of manufacturing it

Additionally the extent of acetylation of the internal mannose on the xanthan side chain and the extent of the pyruvylation of the terminal mannose may vary The present inventors believe that a fully acetylated and/or fully pyruvylated xanthan will have improved rheological properties for certain oil recovery purposes

SUMMARY OF THE INVENTION

An object of the present invention is to provide a family of polysaccharide polymers which are better viscosifiers of water than naturally-occurring xanthan gum Another object of the present invention is to provide a family of polysaccharide polymers having improved rheological properties over naturally-occurring xanthan gum at elevated temperatures and/or in the presence of salts and which members possess other desired properties

It is also an object of the present invention to provide an in vitro method for obtaining certain of these products and microorganisms having the ability to produce members of this family of polysaccharide polymers in vivo A further object of the present invention is to provide processes for preparing members of this family of polysaccharides by aerobically fermenting microorganisms having the ability to produce the various polysaccharide polymers

Additional objects and advantages of the invention will be set forth in part in the description which follows. and in part will be obvious from the description. or may be learned by practice of the invention. The objects and advantages may be realized and attained by means of the instrumentalities and combinations particularly pointed out in the appended claims

To further achieve the objects and in accordance with the purposes of the present invention. there is provided a composition comprising xanthan gum wherein the mannose moieties are not acetylated This gum is hereinafter referred to as "non-acetylated xanthan." A second structure is referred to herein where the terminal mannose moieties of xanthan gum are not pyruvylated. This gum will be referred to as "non-pyruvylated xanthan " Moreover. there has been disclosed a gum which is neither acetylated nor pyruvylated. This polymer is hereinafter referred to as "non-acetylated. non-pyruvylated xanthan gum." Also. the present invention relates to a xanthan gum herein referred to as "fully acetylated xanthan gum." wherein at least 90% of the internal mannose moieties are acetylated. preferably 95% and more preferably 100%. are acetylated. Also. the present invention relates to a xanthan gum wherein at least 90% of the terminal mannose moieties. preferably 95% and more preferably 100%. are pyruvylated. herein referred to as "fully-pyruvylated xanthan gum."

This invention also contemplates processes for the production of the polysaccharide polymers described above. The polysaccharide polymers of this invention can be made generally by genetic manipulations of the microbial biosynthetic pathways which lead to the production of polysaccharides. In particular. microbial pathways for the production of xanthan gum may be manipulated to create an in vivo or in vitro system for the production of an altered polymeric unit. Thus. systems can be created through the use of regulatable Acetylase and Ketalase genes in particular. to create polysaccharides which are acetylated or pyruvylated to varying degrees For example. it is contemplated that

xanthan gum which is 10% 20% 30% 40% or 50% can be synthesized as well as xanthan which is 10% 20% 30% 40% 50% 60% 70% or 80% pyruvylated. Microorganisms which produce the present polysaccharide polymers _in vivo_ and methods of using these polysaccharide polymers are also disclosed

Various strains of X campestis described more fully hereinbelow have been deposited with the American Type Culture Collection Rockville Maryland on March 21 1986. These strains are X921 X1006 X934 and X1231 and have been deposited under Accession Nos 53472 53473 53474 and 67344 respectively

It is understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention. as claimed. The accompanying drawings which are incorporated in and constitute a part of this specification illustrate various embodiments of the invention and together with the description. serve to explain the principles of the invention

BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1 depicts the presumed pathway of xanthan gum biosynthesis. Abbreviations used are Glu=Glucose. GluA=GLucuronic acid Man=Mannose Glu-Glu=Cellobiose P=Phosphate PP=Pyrophosphate C55=Isoprenoid Lipid Carrier: PEP=Phosphoenol-pyruvate AcCoA=Acetylcoenzyne A. I-V=Glycosyltransferases. UDP-Uridine 5'-diphosphate: and GDP=Guanosine 5'-Diphosphate

FIGURE 2 depicts a viscosity comparison between wild-type and non-pyruvylated gums.

FIGURE 3 depicts a viscosity comparison between non-acetylated and chemically-deacetylated gums

FIGURE 4 depicts the approximate physical location of 3 TnK12 insertion mutations within the cloned gum gene cluster DNA of recombinant plasmid pRK290-H336. This figure also shows the approximate locations of Spel restriction endonuclease cleavage sites in pRK290-H336

DETAILED DESCRIPTION OF THE INVENTION

Reference will now be made in detail to the presently preferred embodiments of the present invention which. together with the following examples serve to explain the principles of the invention

The polysaccharide polymers of the present invention have been described in detail above. These polysaccharide polymers can be produced _in vitro_ with a cell-free enzyme system or can be produced _in vivo_ by growing cells of an appropriate mutant strain. Other means of preparing the polysaccharide polymers are also described below

In Vitro Polysaccharide Synthesis

The basic method relating to the use of a cell-free systems to make non-variant xanthan gum is described by Ielpi. L. Couso R.O and Dankert. M A. in FEBS Letters 130 253-256 (1981) specifically incorporated herein by reference. It has been found that a modified version of this method may be employed to create the variant polysaccharides of this invention.

For this novel. modified method. the _in vitro_ cell-free system is prepared generally by lysing cells of a microorganism of the genus Xanthomonas. preferably Xanthomonas campestris in the presence of a suitable buffer preferably including EDTA. and obtaining the appropriate biosynthetic enzymes which are able to subsequently process exogenously added substrates Alternate means of lysis may be used including but not limited to sonication French Pressure cell detergent treatment enzyme treatment and combinations thereof

Generally. to produce the variant polysaccharides of the present invention a lysate of a microorganism possessing the enzymes required to assemble the desired polysaccharide is incubated with the appropriate substrates. which. depending on the gum desired. may include UDP-glucose. GDP-mannose. UDP-glucuronic acid. acetyl-CoA and phosphoenolpyruvate. The choice of substrates is dependent on the polysaccharide which it is desired to produce For example. a non-acetylated polysaccharide is obtained by eliminating acetyl-CoA as a substrate Similarly. a non-pyruvylated gum is obtained by eliminating phosphoenolpyruvate as a substrate. Chemical and/or enzymatic treatment of the cell lysates in order to deplete endogeneous substrates will be evident to one skilled in the art.

In addition. cell-free systems may be created from mutant organisms deficient in one or more of the enzymes of the xanthan biosynthetic pathway set forth in Figure 1. Such mutant-derived cell lysates would produce the variant gums described herein. either due solely to the mutation or due to the mutation in combination with a withheld substrate

The biosynthetic process may. in one embodiment. be monitored by the incorporation of radiolabeled substrates into the polymeric units. Other methods may also be used to allow identification of the biosynthetic intermediates that are known to those of ordinary skill in the art. In particular. chromatographic methods have been developed to separate and to identify the oligosaccharide intermediates. These include thin layer chromatography and high-performance liquid chromatography

The cell-free biosynthesis of xanthan has been found to be a time-dependent. sequential process that is dependent

on the addition of all three specific sugar nucleotides The background of non-specific incorporation of labeled substrate is minimal and does not interfere with the detection of the xanthan-specific polymer in the gum fraction

The involvement of lipid carriers specifically isoprenoid pyrophosphate has been shown in several polysaccharide biosynthetic pathways Additionally the involvement of pyrophosphoryl-linked lipid carrier in xanthan biosynthesis has been demonstrated Thus the xanthan biosynthetic intermediates have been found to be recoverable in the organic soluble fraction with these carrier lipids The recovered oligosaccharide can subsequently be freed from the carrier lipid by mild acid hydrolysis for example pH 2 for 20 minutes at 90°C and dephosphorylated with alkaline phosphatase for analysis

Using these methods for recovery of intermediate products it has been discovered that under in vitro conditions certain lysates of X campestris mutants will produce non-acetylated or non-pyruvylated xanthan gum even in the presence of all substrates required for non-variant gum synthesis In light of the teachings herein these methods will enable one skilled in the art to identify cell lysates which produce other altered polysaccharides

In Vivo Polysaccharide Synthesis

The development of the cell-free synthesis process for the polysaccharides described above demonstrated that various Xanthomonas campestris cells have all the enzymes necessary to synthesize acetylated or non-acetylated polytetramer. non-acetylated and/or non-pyruvylated xanthan gum and fully-acetylated xanthan gum

Furthermore for whole cells to synthesize non-acetylated xanthan gum a means of blocking the acetylation step during xanthan gum synthesis would be required Additionally for the whole cells to synthesize non-acetylated. non-pyruvylated xanthan gum a means of blocking xanthan gum synthesis at both the acetylation and pyruvylation steps would be required In one embodiment of the present invention mutagenesis was employed to alter some of the genes responsible for these various reactions

Transposons. including but not limited to Tn10 and Tn903 can be used to mutagenize Xanthomonas campestris. These transposons in one embodiment confer resistance to tetracycline and kanamycin respectively Transposons have the ability to insert themselves into genes wherein they cause mutations by interrupting the coding sequence The transposons can be introduced into Xanthomonas campestris on various vectors including on so-called suicide vectors such as pRK2013 Vector pRK2013. as described by Ditta. G Corbin D and Helinski. D R. in Proc Natl Acad. Sci U S A. 77 7347-7351 (1980). specifically incorporated herein by reference has the ability to transfer itself into non-enteric bacteria. such as Xanthomonas campestris. but cannot replicate in that host. Thus. if the suicide vector is introduced into a population of Xanthomonas campestris cells and that population is subsequently challenged with either tetracycline or kanamycin. the individuals which survive are those in which one of the transposons has inserted into the genome of Xanthonomas campestris' Survivors of such a challenge can be screened for those which have lost the ability to make xanthan gum Such mutants may appear less mucoid than wild-type Xanthomonas campestris

In other embodiments of the invention. other means of mutagenesis can be employed to generate mutants which have lost the ability to make xanthan gum or that do not acetylate and/or pyruvylate the gums they produce Such means will readily occur to one skilled in the art. and include. without limitation. irradiation. recombinant DNA technology (in particular. as described in United States Patent Application Serial No. 842.944 of Capage et al entitled "Recombinant-DNA Mediated Production of Xanthan Gum." filed March 26. 1986. and incorporated specifically herein by reference. and in Example 1 below) and chemical mutagen treatment Examples of such mutagenesis procedures have been described by Miller J H in Experiments in Molecular Genetics (1972). Davis R.W Bostein D and Roth. J E in Advanced Bacterial Genetics (1980) and Maniatis T. Fritsch E F and Sambrook J in Molecular Cloning (1982). Cold Spring Harbor.

Although mutants can first be chosen which appear less mucoid than wild-type organisms. those desired generally retain the ability to make some polysaccharide Cell-free extracts of each of the xanthan mutants can be prepared and tested as noted above by the addition of different combinations of substrates and analysis of the resultant products

Alternatively. appropriate mutants can be detected by assaying the culture broth of each mutant for the presence of the desired polysaccharide. e.g. xanthan gum which is non-acetylated. non-acetylated and non-pyruvylated. low pyruvate or fully-acetylated. Thus. mutants can be found which appear to be blocked at various positions of the xanthan gum pathway. A mutant of Xanthomonas campestris which produces non-acetylated xanthan gum (X1006). a mutant which produces non-pyruvylated gum (X921). a mutant which produces xanthan gum containing low levels of pyruvylation (less than 5% of the terminal mannose X934). and a mutant which produces non-acetylated. non-pyruvylated xanthan gum (X1231 (p41KS22)) have each been placed on deposit at the American Type Culture Collection. Rockville. Maryland. under Accession Nos. 53472. 53473. 53474 and 67344. respectively

It is not beyond the scope of the invention to employ enzyme inhibitors of wild-type. Acetylase and Ketalase to arrive at the same products Still other alternatives for producing this family of polysaccharides are contemplated. including enzymatic and chemical degradation of natural xanthan gum.

The mutants can be grown under conditions generally known in the art for growth of wild-type Xanthomonas camp-

estris. For example they can be grown on suitable assimilable carbon sources such as glucose sucrose maltose starch invert sugar complex carbohydrates such as molasses or corn syrup various organic acids and the like. Mixtures of carbon sources can also be employed. The concentration of carbon source supplied is often between 10 and 60 grams per liter. Also necessary for growth are an assimilable source of organic or inorganic nitrogen generally between about 0.1 and 10.0 grams per liter, and minerals, the choice of which are easily within the skill of the art. Examples of suitable nitrogen sources are ammonium salts, nitrate, urea, yeast extract, peptone, or other hydrolyzed proteinaceous materials or mixtures thereof. Examples of suitable minerals include phosphorus, sulfur, potassium, sodium, iron, magnesium; these are often added with a chelating agent such as EDTA or citric acid.

Optimal temperatures for growth of Xanthomonas campestris generally are between 18°C and 35°C preferably between about 27°C and 30°C. Xanthomonas campestris cells are grown aerobically by supplying air or oxygen so that an adequate level of dissolved oxygen is maintained, for example above about 10% of saturation. Preferably, the level is kept above about 20%. The pH often is maintained at about 6.0 to 8.0 preferably at about 6.5 to 7.5.

The polysaccharides of the present invention can be recovered from fermentation broths by a suitable means. Precipitation with isopropanol, ethanol, or other suitable alcohol readily yields the polysaccharides of this invention. Generally, alcohols are added to a concentration of about 50 to 75% on the basis of volume, preferably in the presence of potassium chloride, sodium chloride or other salt. Alternatively the polymers can be recovered from the broth by ultra-filtration.

Non-pyruvylated xanthan gums are superior to xanthan as viscosifiers of aqueous mediums in particular applications. The viscosity of solutions of non-acetylated xanthan and non-pyruvylated xanthan are retained at conditions of high temperature and/or high salinity. The products of this invention are thus ideally suited for use in secondary and tertiary oil recovery.

Mobility control solutions for use in enhanced oil recovery may also be prepared from the variant polysaccharide polymers disclosed herein. Solutions of the polysaccharide polymers at concentrations of from about 50 to about 3000 ppm are appropriate for such mobility control solutions. Other known additives may also be used in combination with these solutions to further enhance oil recovery. Such additives include, for example, surfactants, alkaline agents or metal or organic crosslinking agents.

The polysaccharide polymers, like xanthan gum, can also be used as thickening agents in foods, cosmetics, medicinal formulations, paper sizings, drilling muds, printing inks, and the like and as a gelling agent. In addition they can be used to reduce frictional drag of fluid flow in pipes.

EXAMPLE 1

This example shows the methods of mutagenesis and screening employed to generate X. campestris mutant strains having defects in Acetylase or Ketalase activity.

The genes encoding the enzymes of xanthan gum biosynthesis have been shown to comprise a set of clustered genes on the X. campestris chromosome. This "gum gene cluster" has been described in detail by Capage et al. Segments of gum gene DNA have been cloned on plasmid vectors such as pMW79 as detailed in Capage et al.

Regionally-directed mutagenesis was performed upon subcloned portions of the gum DNA carried in plasmid pMW79. These cloned DNA segments were mutagenized in vivo with transposons and in vitro by using recombinant DNA technology to generate insertion, deletion, and substitution mutations within the cloned X. campestris DNA. In order to study the phenotypes conferred by these mutations, the plasmids carrying the mutations were transferred back into X. campestris and subsequently recombinants were identified in which the plasmid-borne, mutated gene had been inserted in the chromosome via homologous recombination. The tetracycline resistance encoded by Tn10 affords a convenient selective system for movement of mutations from a plasmid into the chromosome.

One such mutant strain (X1006) carried a Tn10 insertion that was found to cause inactivation of the Acetylase activity. This mutant strain was characterized as described in Example 2 and 3, and found to produce a polysaccharide that was non-actylated. A second mutant strain was constructed by the in vitro insertion of a fragment of DNA containing the tetracycline resistance gene of Tn10 into a restriction site within the gum gene cluster. This mutant strain (X921) was found to be defective in the Ketalase activity. As found by the methods of Examples 2 and 3, this mutant produced xanthan that was non-pyruvylated.

A third mutant strain (X934) was also found that greatly reduces the ketalase activity. This mutant strain produces xanthan gum that has a very low level of pyruvylation: 1-5% of the level of pyruvylation found in normal xanthan.

The mutant strain X934 was found as described below. In preliminary experiments designed to study recombination between plasmid-borne X. campestris DNA and the X. campestris chromosome the plasmid pTX655 was used as a model system. This plasmid carries a Tn10 insert in the middle of a 2.3 kb X. campestris PstI fragment cloned in plasmid RSF1010. This insertion of Tn10 causes the Gum- defect in the mutant strain X655 as described by Capage et al. and Vanderslice et al. The experiment was to mobilize pTX655 with plasmid pRK2013 and transfer it from E. coli into X. campestris by selecting for transfer of the tetracycline resistance encoded by Tn10. The initial results of this mating

were anomalous and suggested that Tn10 did not express tetracycline resistance efficiently in X campestris when carried on the plasmid but that the drug resistance was more efficiently expressed when Tn10 was carried in the chromosome of X campestris This phenomenon has also been described for Tn10 in E coli. There it has been shown that strains carrying one copy of Tn10 inserted in the chromosome are resistant to significantly higher concentrations of tetracycline than are strains carrying Tn10 on a multicopy plasmid The selection of Tet' X campestris out of the above mating resulted in a high frequency (0 5 per recipient) of progeny which grew very poorly i e only small watery colonies on tetracycline After prolonged incubation a large fraction of the colonies (25%) produced sectors of more vigorously growing cells More than 50% of these sectors appeared to be Gum- in morphology These probably result from recombination between the plasmid-borne DNA containing the Tn10 insertion and the chromosomal wild type DNA When the Tn10 is recombined into the chromosome high-level Tet' is obtained and the vigorously growing sector is observed When these Gum- Tet' sectors were picked and restreaked on tetracycline they grew well and displayed a characteristic Gum- morphology

Gum- Tet' isolates were also characterized Some of these strains (in particular X934) were found to contain the entire plasmid pTX655 inserted into the chromosome of X campestris via homologous recombination The chromosomal structure of the X934 strain was determined by Southern blot hybridization of the chromosomal DNA which shows that the plasmid sequences exist in a chromosomally integrated form.

EXAMPLE 2

This example shows how the altered polysaccharides of the present invention can be prepared in vitro For instance it shows how non-acetylated and/or non-pyruvylated xanthan gum was prepared in vitro

Preparation of Lysates

Xanthomonas campestris B1459 S4-L or S4-L mutants described in Examples 1 and 5 were grown in YM (yeast-malt medium) supplemented with 2% (w/v) glucose as described by Jeanes A. et al in U S Department of Agriculture ARS-NC-51 pp 14 (1976) specifically incorporated herein by reference Cultures were grown to late log phase at 30°C The cells were harvested by centrifugation and washed with cold Tris-HCl 70mM pH 8 2 with 10mM EDTA and were freeze-thawed three times by a procedure similar to Garcia. R C et al described in European Journal of Biochemistry 43 93-105 (1974). specifically incorporated herein by reference This procedure ruptured the cells. as was evidenced by the increased viscosity of the suspensions and the complete loss of cell viability (one of 10^6 survivors) after this treatment The freeze-thawed lysates were frozen in aliquots at -80°C Protein concentration was determined with BIO RAD assay (BIO RAD Laboratories Richmond California) and was found to be 5 to 7 mg cell protein per ml of lysate

Biosynthetic Assay Procedure

As described by Ielpi. L.. Couso. R O . and Dankert. M.A. in FEBS Letters 130 253-256 (1981). specifically incorporated herein by reference. an aliquot of freeze-thawed lysate (equivalent to 300 to 400 ug protein). DNAase I (10 ug/ml) and $MgCl_2$ (8 mM) were preincubated at 20°C for twenty minutes An equal volume of 70 mM Tris-HCl. pH 8 2. with the desired radiolabeled sugar nucleotides (UDP-glucose. GDP-mannose and UDP-glucuronic acid) were added and incubated at 20°C Radiolabeled phosphoenol pyruvate and acetyl coenzyme A were added when desired as described in Ielpi et al supra. and Ielpi L Couso. R O. and Dankert M A Biochem Biophys Res. Comm. 102 1400-1408 (1981) and Ielpi. L.. Couso R.O.. and Dankert. M.A.. Biochem. Intern 6 323-333 (1983). both of which are specifically incorporated herein by reference. At various times. the reactions were stopped by dilution with 4°C buffer. The samples were centrifuged and the pellets were washed two times with buffer The supernatants were combined. carrier xanthan (100 ug) was added. and the xanthan plus synthesized polymer were precipitated with ethanol (60%)-KCl(0 8%). The precipitated polymer was resuspended in water and reprecipitated two more times to remove unincorporated label. Radioactivity incorporated into the precipitate (termed the gum fraction) was determined in a liquid scintillation counter and the data were processed to obtain incorporation in terms of picomoles of the radiolabeled components

Cell lysates of X1006 did not incorporate carbon-14 acetate from [^{14}C] acetyl CoA into the gum fraction of the in vitro system. Cell lysates of S4-L did produce in vitro gum radiolabeled with [^{14}C] acetate. Similarly. cell lysates of X921 did not incorporate [^{14}C] pyruvate into the gum fraction while S4-L cell lysates did incorporate radiolabeled pyruvate from phosphoenoL [^{14}C] pyruvate into the gum fraction of the in vitro system. Thus. X1006 was identified as a mutant strain with a defect in the gene for Acetylase and X921 as a mutant strain with a defect in the gene for Ketalase. Lysates of these strains produced non-acetylated xanthan and non-pyuvylated xanthan. respectively. in vitro.

It has also been shown that. by withholding substrates. X campestris B1459 S4-L lysates produce altered polysac-

charides in vitro. For example, cell lysates of S4-L did produce non-acetylated, non-pyruvylated xanthan gum in vitro when the endogenous acetyl-CoA and phosphoenolpyruvate were depleted.

EXAMPLE 3

This example demonstrates the use of the Acetylase-deficient strain X1006 to produce non-acetylated gum in vivo. This example also demonstrates the in vivo production of the non-pyruvylated gum from the Ketalase-minus strain X921 and xanthan gum with a reduced level of pyruvylation from the strain X934.

The three mutant strains described above and S4-L were grown overnight in broth with two percent glucose at 30°C. The gum was harvested by removing cells by centrifugation, precipitating the gum from the supernatant by addition of 2-propanol or ethanol with 0.5 to 1% potassium chloride. The gum precipitate was recovered by centrifugation and resuspended. The procedure was repeated. The resuspended gum was dialysed against water. A sample of each polysaccharide was acid hydrolyzed and analyzed by HPLC using a BIO RAD HPX-87H column. Xanthan components were quantitated by the injection of standards of known concentration.

The HPLC analysis of the hydrolyzed gums showed that X921 produced xanthan gum without pyruvate. Strain X1006 produced xanthan gum with no acetate. Strain X934 produced a gum that contained pyruvate at a level of 1-5% that of S4-L gum.

EXAMPLE 4

This example describes methods and strategies that could be employed to construct mutants of X. campestris that will produce xanthan that is both non-acetylated and non-pyruvylated.

Mutant strains of X. campestris lacking Ketalase (X921) or Acetylase (X1006) activity have been described. One could use microbial genetics and recombinant DNA methods as described by Vanderslice et al and Capage et al to introduce a Ketalase defect and an Acetylase defect into a single strain of X. campestris. This double-mutant strain would produce xanthan gum that was both non-acetylated and non-pyruvylated.

Methods for producing insertion mutations into cloned gum gene DNA carried on plasmid vectors have been described by Capage et al. One could envision using a plasmid carrying the insertion mutation that gave rise to mutant strain X921 as a substrate for a second round of mutagenesis in vivo or in vitro. This second round of mutagenesis could employ any of a number of transposable elements that would readily occur to one skilled in the art or any of an equally obvious number of DNA restriction fragments containing selectable markers. A set of plasmids carrying two insertions mutations could be used in the gene replacement technique of Capage et al to transfer the plasmid-borne mutations into the chromosome of X. campestris. Phenotypes of the resultant strains can be analyzed by the in vivo and in vitro techniques described by Vanderslice et al and Capage et al supra in Example 2. These analyses will reveal doubly mutant strains that are blocked in both the Ketalase and Acetylase activity. These double mutant strains will produce xanthan that is simultaneously non-pyruvylated and non-acetylated.

EXAMPLE 5

This example discusses cloning the acetylase gene and the Ketalase gene onto vectors to ensure that the polysaccharides described herein are fully acetylated, fully pyruvylated, or both.

The X. campestris strains X1006 and X921 have mutations in the genes for acetylase and ketalase, respectively, as described in Examples 2 and 3. These mutants were created employing the methods described in Example 1. It is possible for one skilled in the art to employ methods described in Betlach et al supra, to recover native DNA restriction fragments containing the Acetylase or Ketalase genes. That is, plasmids with DNA restriction fragments containing the Acetylase or Ketalase genes interrupted by a drug resistance marker can be used to probe lambda genomic libraries to obtain native DNA sequences for the Acetylase or Ketalase gene. In another embodiment, the genes for the acetylase and ketalase enzymes have already been cloned onto the plasmid pRK290-H336, as described by Capage et al., supra, and other plasmids described therein. It is a simple matter for those skilled in the art to subclone the Acetylase and Ketalase genes themselves from these plasmids.

The native DNA sequences obtained by either method can then be inserted onto plasmids capable of replication in X. campestris, for instance pMW79, using the methods revealed in Betlach et al. and Capage et al. Expression of the Acetylase and/or Ketalase gene can be controlled by modifying the existing DNA sequence or inserting regulatable promoters upstream from the gene. Such techniques are well known to those versed in molecular biology and genetics. Similarly, the plasmids onto which the genes are inserted can be high copy number plasmids. As revealed in Capage et al., the xanthan biosynthetic enzymes are present in low amounts in X. campestris. Insertion of the plasmids described above into X. campestris, and growth of cultures under appropriate conditions for expression of the plasmid-borne genes, will result in synthesis of much greater numbers of the Acetylase and/or Ketalase enzymes than the other

xanthan biosynthetic enzymes Overexpression of the Acetylase should cause the xanthan polysaccharide to be fully acetylated i e all internal mannose residues acetylated Similarly overexpression of the Ketalase should cause xanthan polysaccharide to be fully pyruvylated on the terminal mannose

It should be evident to one skilled in the art that full acetylation and full pyruvylation of xanthan can be achieved by the methods described above

EXAMPLE 6

This example demonstrates the economic and technical advantages of a non-pyruvylated polysaccharide produced by a genetically modified Xanthomonas campestris for viscosifying water at high temperatures

Xanthan gum a natural product of Xanthomonas campestris is an effective viscosifier of water for use in for example enhanced recovery of petroleum These viscosity applications frequently necessitate xanthan gum to be applied at high temperature and in saline brines Xanthan gums are effective viscosifiers even at high temperature (for example 75° to 100°C) although their viscosity is substantially reduced over that at lower temperature (for example, 25° to 60°C)

The inventors have discovered a new and novel polysaccharide produced by a genetically modified strain of Xanthomonas campestris (strain X921) that produced a viscosity at high temperature equal to that of wild-type xanthan gum produced from the S4-L parent strain (strain X237) This xanthan gum has the normal pentameric xanthan structure but, because of the genetic modification, contains no pyruvate moiety on the terminal mannose This novel polysaccharide has low viscosity at fermentation temperature near 30°C which will result in substantial cost savings and processing conveniences The cost to produce pyruvylated xanthan gum is high primarily because the polymer's high viscosity requires great energy input for agitation aeration and cooling

Figure 2 shows a viscosity comparison of the novel non-pyruvylated xanthan gum to a wild-type xanthan gum produced by the unmodified parent The wild-type gum shows high viscosity at low temperature but the viscosity decreases rapidly with increasing temperature The non-pyruvylated gum, on the other hand, has lower viscosity at low temperature but retains a viscosity at high temperature essentially equivalent to the wild-type xanthan gum The viscosities reported in Figure 2 were recorded at a shear rate of 8 s⁻¹ in a concentric cylinder viscometer on solutions of 1000 ppm active polymer solids in 5000 ppm NaCl brine

EXAMPLE 7

This example demonstrates the advantages of a non-acetylated xanthan produced by a genetically modified Xanthomonas campestris for use as a visosifying agent for aqueous solutions

Figure 3 compares the viscosities of chemically deacetylated commercial xanthan and its parent compound with those of a non-acetylated xanthan polysaccharide made from a genetically manipulated Xanthomonas campestris (strain X1006) and of the xanthan gum made from the wild-type X. campestris parent (strain X237) The viscosities were obtained at a shear rate of 8 s⁻¹ for 1000 ppm polymer concentration in 50 000 ppm NaCl brine Viscosities were measured over the temperature range of 25° to about 80°C

Figure 3 demonstrates that chemical deacetylation of xanthan results in a loss of viscosifying power over the entire temperature range However, elimination of acetylation by genetic means results in substantially increased viscosity compared to the wild-type xanthan gum Thus non-acetylated xanthan gum produced by a mutant strain of X campestris is an improved polysaccharide compared to the xanthan itself and compared to deacetylated xanthan gum produced by chemical methods

Example 8

This example describes the methods used to construct a double mutant strain of X. campestris that produces non-acetylated, non-pyruvylated xanthan.

Capage et al. have described the cloning of a gene cluster from X. campestris that contains genes that direct the biosynthesis of xanthan gum. They also described the isolation of chromosomal deletion mutations in X campestris that eliminate all or varying portions of this gene cluster. One such deletion mutant, strain X1231, lacks all of the X. campestris DNA that is carried on the recombinant plasmid pRK290-H336. Thus strain X1231 does not synthesize xanthan. When pRK290-H336 is transferred into strain X1231, the ability to synthesize xanthan is restored Capage et al. also described methods of isolating and characterizing insertion mutations of transposon TnK12 within the cloned gum gene DNA carried on pRK290-H336. Two such mutant plasmids are pRK290-H336.22 and pRK290-H336 41. The approximate locations of the TnK12 inserts on each of these plasmids is shown in Figure 4. When pRK290-H336 22 is transferred into X1231, the resultant strain produces non-acetylated xanthan When pRK290-H336.41 is transferred into X1231, non-pyruvylated xanthan is produced. These two plasmid mutants have been used to construct, by means

on in vitro recombination a double mutant plasmid that directs synthesis of non-acetylated non-pyruvylated xanthan when carried in the deletion strain X1231

The in vitro strategy for generating a non-acetylated non-pyruvylated double mutant plasmid is as follows A kanamycin-sensitive (Kan⁵) derivative of pRK290-H336 41 was generated by doing a partial HindIII digestion of the plasmid ligating the digestion products at very low DNA concentrations (to promote intramolecular ligation) and then screening tetracycline-resistant (Tet') transformants for kanamycin sensitivity Plasmid DNAs were then prepared from Tet' Kan⁵ isolates and analyzed by restriction endonuclease digestion and agarose gel electrophoresis There are only three HindIII sites in pRK290-H336 41 and two of these occur within TnK12 and bracket the Kan gene Thus a high proportion of the deletions generated in the partial digestion were deleted for the Kan gene whereas the rest of the plasmid was retained intact. The Kan⁵ plasmid still carries an insertion (1 kb) in the ketalase gene and thus was expected to yield non-pyruvylated gum This plasmid is termed p41KS The next step was to clone the large SpeI fragment of the non-acetylated mutant plasmid pRK290-H336 22 into p41KS As shown in Figure 4 each plasmid contains three SpeI sites at positions 758 771 and 11 716 within the DNA sequence of Capage et al The 10 9 kb SpeI fragment carries the Tnk12 insertion of pRK290-H336 22 The small (13 bp) SpeI fragment lies entirely within a tRNA gene which which is nonessential for X campestris growth and xanthan production Thus deletion of this small SpeI segment in the process of the double mutant construction ought not to affect xanthan biosynthesis Plasmids p41KS and pRK290-H336.22 were purified and digested to completion with SceI and a ligation was performed In this ligation p41KS/SpeI was ligated in 10x molar excess with H336 22/SpeI Thus when recombinants containing the Kan' SpeI fragment of H336 22 were selected. they should most often be associated with the SceI vector fragment of p41KS We performed transformations with these ligations and obtained Kan' transformants. The plasmids carried by these transformants were analyzed to identify the recombinants of interest. The desired recombinant plasmid was readily identified among the Kan' transformants This recombinant plasmid. termed p41KS22 contains the p41KS-derived insertion in the ketalase gene and the H336 22-derived TnK12 insertion within the acetylase gene Appropriate restriction digestion analysis confirmed the presence of both insertion mutations and furthermore showed that the SpeI fragment containing the TnK12 mutation had been inserted in the correct orientation

Plasmid p41KS22 was subsequently transferred into a series of X campestris strains via conjugation The large Gum⁻ deletion strain X1231 was among the recipients This deletion lacks all of the gum gene DNA carried on p41KS22 therefore. X1231 carrying p41KS22 should produce non-acetylated. non-pyruvylated xanthan The plasmid transferred efficiently into X1231 and the resultant phenotype was clearly mucoid but significantly less so than a wild-type control Polysaccharide produced by X1231 carrying p41KS22 was prepared and analyzed This polymer contained glucose. mannose and glucuronic acid but no detectable acetate or pyruvate demonstrating that X1231 (p41KS22) does produce the expected non-acetylated non-pyruvylated gum

Example 9

This example describes the construction and properties of a double mutant plasmid that combines an Acetylase mutation and a Transferase IV mutation.

Capage et al described methods for isolating and characterizing transposon TnK12 insetions within the cloned gum gene DNA carried by plasmid pRK290-H336. One mutant plasmid carrying such an insertion is pRK290-H336 6 The approximate location of the TnK12 insertion in this plasmid is shown in Figure 4. When present in the deletion strain X1231 this plasmid directs the synthesis of polytrimer gum as a result of the insertional inactivation of Transferase IV Using procedures analogous to those described in Example 8. a double mutant plasmid was constructed that combines this Transferase IV defect with the Acetylase mutation carried in pRK290-H336 22. A kanamycin-sensitive derivative of pRK290-H336 6 was derived by deletion of the HindIII fragment of TnK12. This plasmid. p6KS. is analogous to the Kan⁵ plasmid p41KS and still retains 1 kb of TnK12 inserted within the Transferase IV gene Subsequently. the large TnK12-containing SpeI fragment of pRK290-H336 22 was ligated into SpeI-digested p6KS plasmid DNA as described in Example 8 and the double mutant plasmid p6KS22 was obtained This plasmid carries insertion mutations in Transferase IV and the Acetylase When transferred into deletion strain X1231. it ought to direct the synthesis on non-acetylated polytrimer. However. in several independent plasmid transfer experiments. no transfer of p6KS22 into strain X1231 was detected. although the plasmid was successfully transferred at high frequency into other recipients. including both Gum⁻ and Gum⁺ strains. This result suggests that the presence of p6KS22 in strain X1231 is lethal. probably as a direct result of production of non-acetylated polytrimer gum. Capage et al. described three other lethal mutations within the gum gene cluster and concluded that these lethal mutations cause the accumulation of a toxic intermediate in xanthan biosynthesis Accumulation of non-acetylated polytrimer could potentially be toxic if this polysaccharide cannot be secreted by the transport system that normally secretes xanthan.

It is to be understood that application of the teachings of the present invention to a specific microorganism will be within capabilities of one having ordinary skill in the art in light of the teachings contained herein.

It will be apparent to those skilled in the art that various modifications and variations can be made in the processes

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and products of the present invention. Thus, it is intended that the present invention cover the modifications and variations of this invention provided they come within the scope of the appended claims

Claims

1. A water-soluble polysaccharide polymer comprising repeating pentamer units having a D-glucose D-mannose D-glucuronic acid ratio of about 2:2:1 wherein the D-glucose moieties are linked in beta-[1 4] configuration inner D-mannose moieties are linked in an alpha-[1 3] configuration primarily to alternate glucose moieties. the D-glucuronic acid moieties are linked in a beta-[1 2] configuration to said inner mannose moieties and outer mannose moieties are linked to said glucuronic acid moieties in a beta-[1 4] configuration wherein said polysaccharide polymer is acetylated and not pyruvylated

2. A process for preparing a water-soluble polysaccharide polymer comprising repeating pentamer units having a D-glucose D-mannose D-glucuronic acid ratio of about 2:2:1 wherein the D-glucose moieties are linked in beta-[1 4] configuration inner D-mannose moieties are linked in an alpha-[1 3] configuration primarily to alternate glucose moieties the D-glucuronic acid moieties are linked in a beta-[1 2] configuration to said inner mannose moieties and outer mannose moieties are linked to said glucuronic acid moieties in a beta[1 4] configuration wherein said polysaccharide polymer is acetylated and not pyruvylated said process comprising:

   (a) obtaining a ketalase deficient mutant of *Xanthomonas* and
   (b) culturing said *Xanthomonas* under conditions sufficient to produce said polysaccharide polymer

3. The process of claim 2 wherein said *Xanthomonas* is *Xanthomonas campestris*

4. The process of claim 3 wherein said *Xanthomonas campestris* is X921 having accession number ATCC 53472

5. A mutant *Xanthomonas* capable of producing the polysaccharide polymer as defined in claim 1

6. The mutant *Xanthomonas* of claim 5 wherein said *Xanthomonas* is a ketalase deficient mutant of *Xanthomonas*

7. The mutant *Xanthomanas* of claim 6 wherein said *Xanthomonas* is *Xanthomonas campestris*.

8. The mutant *Xanthomonas* of claim 7 wherein said *Xanthomonas* is *Xanthomonas campestris* X921 having accession number ATCC 53472 .

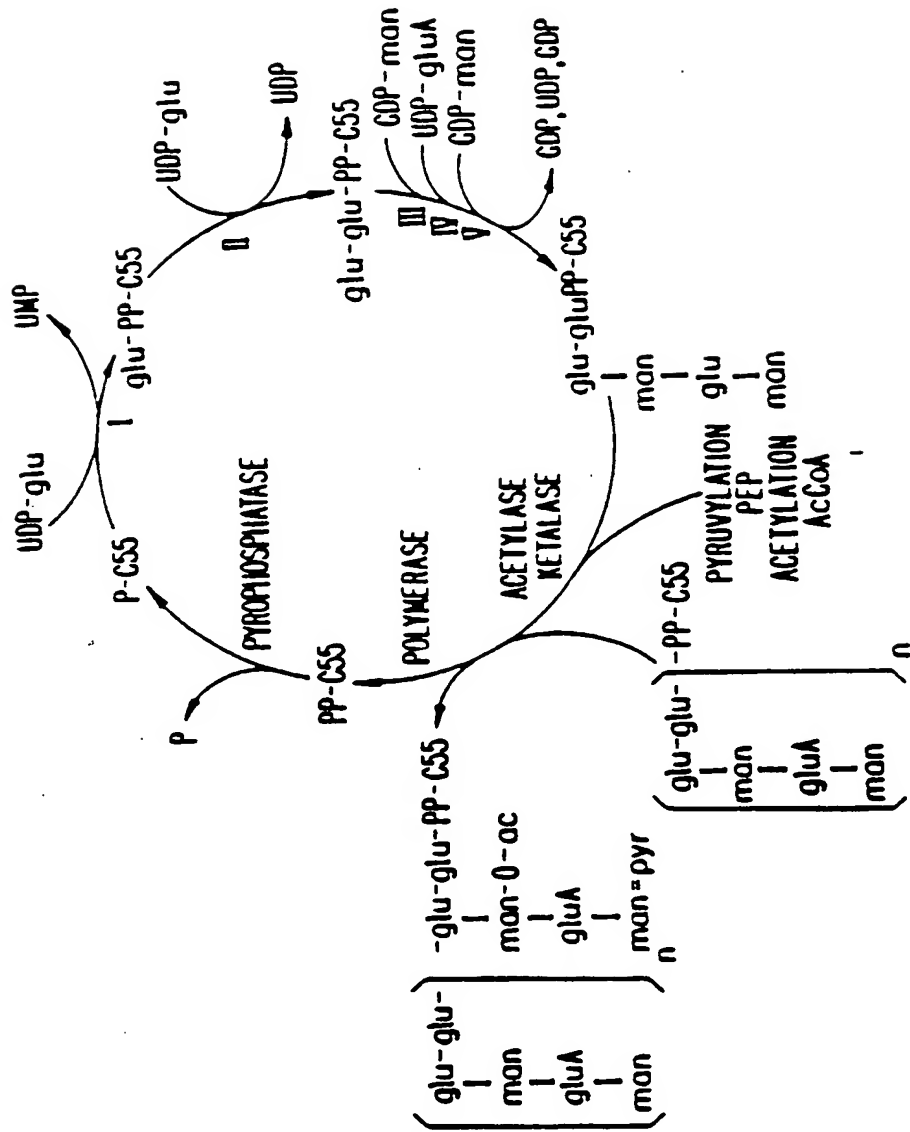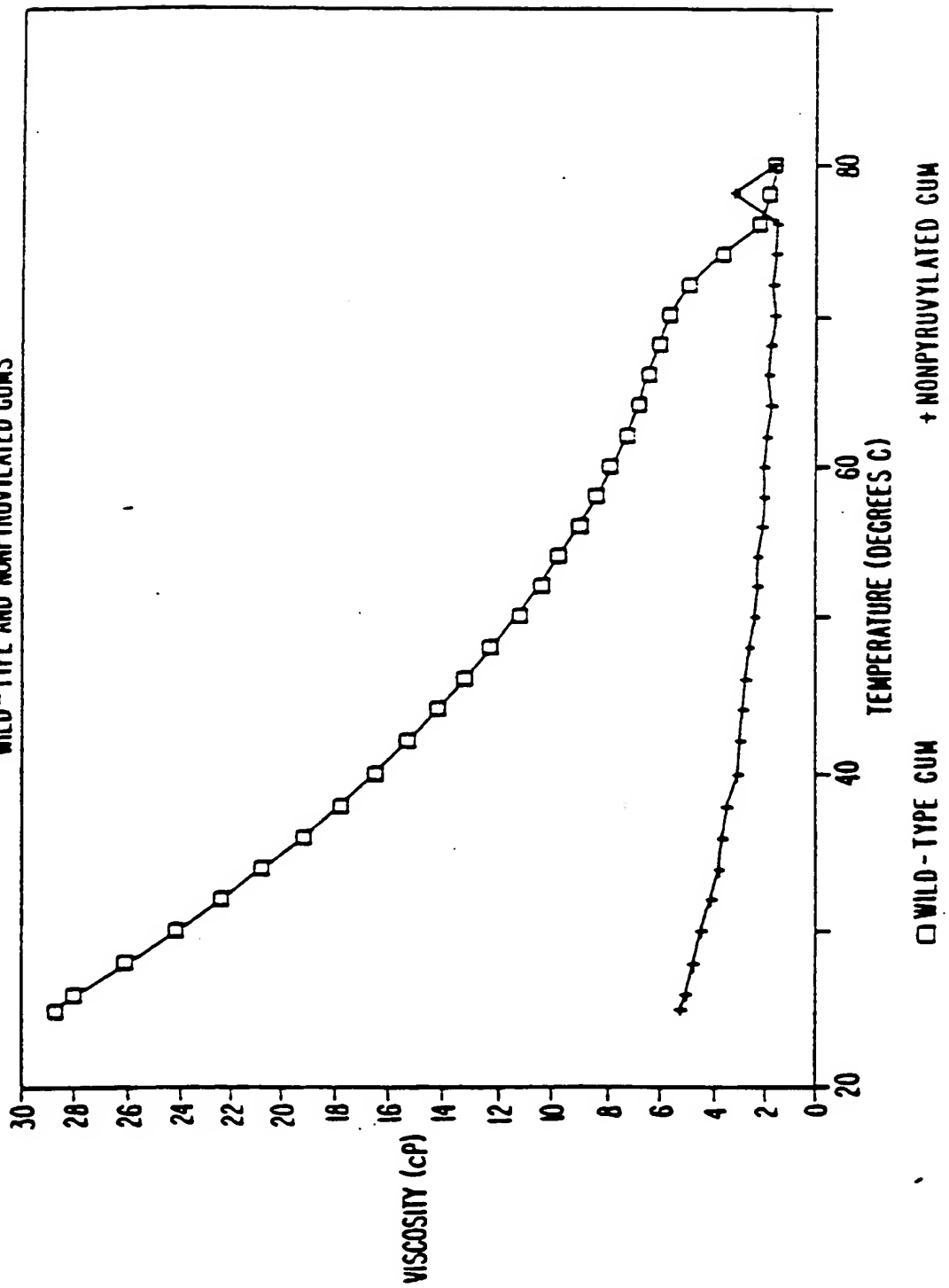9. A water-soluble polysaccharide polymer comprising repeating pentamer units having a D-glucose D-mannose D-glucuronic acid ratio of about 2:2:1. wherein the D-glucose moieties are linked in beta-[1.4] configuration. inner D-mannose moieties are linked in an alpha-[1.3] configuration primarily to alternate glucose moieties. the D-glucuronic acid moieties are linked in a beta-[1.2] configuration to said inner mannose moieties. and outer mannose moieties are linked to said glucuronic acid moieties in a beta-[1 4] configuration wherein said polysaccharide polymer is acetylated and has a level of pyruvylation less than native xanthan

10. The water-soluble polysaccharide polymer of claim 9 wherein 1-5% of said outer mannose moieties of said polysaccharide are pyruvylated.

11. A process for preparing a water-soluble polysaccharide polymer comprising repeating pentamer units having a D-glucose:D-mannose:D-glucuronic acid ratio of about 2:2:1. wherein the D-glucose moieties are linked in beta-[1 4] configuration. inner D-mannose moieties are inked in an alpha-[1 3] configuration primarily to alternate glucose moieties. the D-glucuronic acid moieties are linked in a beta-[1.2] configuration to said inner mannose moieties. and outer mannose moieties are linked to said glucuronic acid moieties in a beta[1.4] configuration. wherein said polysaccharide polymer is acetylated and has a reduced level of pyruvylation. said process comprising:

   (a) obtaining a mutant of *Xanthomonas* having reduced ketalase activity: and
   (b) culturing said *Xanthomonas* under conditions sufficient to produce said polysaccharide polymer.

12. The process of claim 11. wherein said *Xanthomonas* is *Xanthomonas campestris*.

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13. The process of claim 12 wherein said *Xanthomonas campestris* is X934 having accession number ATCC 53474

14. A mutant *Xanthomonas* capable of producing the polysaccharide polymer as defined in claims 9 or 10

15. The mutant *Xanthomonas* of claim 14 wherein said *Xanthomonas* is a reduced-ketalase-activity-mutant of *Xanthomonas*

16. The mutant *Xanthomonas* of claim 15 wherein said *Xanthomonas* is *Xanthomonas campestris*

17. The mutant *Xanthomonas* of claim 16 wherein said *Xanthomonas* is *Xanthomonas campestris* X934 having accession number ATCC 53474

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FIG. 1.

FIG. 2.
VISCOSITY COMPARISON
WILD-TYPE AND NONPYRUVYLATED GUMS

FIG. 3.

VISCOSITY COMPARISON
NONACETYLATED VERSUS DEACETYLATED GUMS

FIG. 4.

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(54) **Family of xanthan-based polysaccharide polymers including non-acetylated and/or non-pyruvylated gum**

(57) Variant xanthan gums, including non-acetylated, non-pyruvylated, non-acetylated and non-pyruvylated, and fully-acetylated xanthan gums. In addition, in vitro and in vivo methods for the synthesis of these gums are disclosed. Mutant **Xanthomonas campestris** strains useful in these synthesis are also specified.

FIG. I.
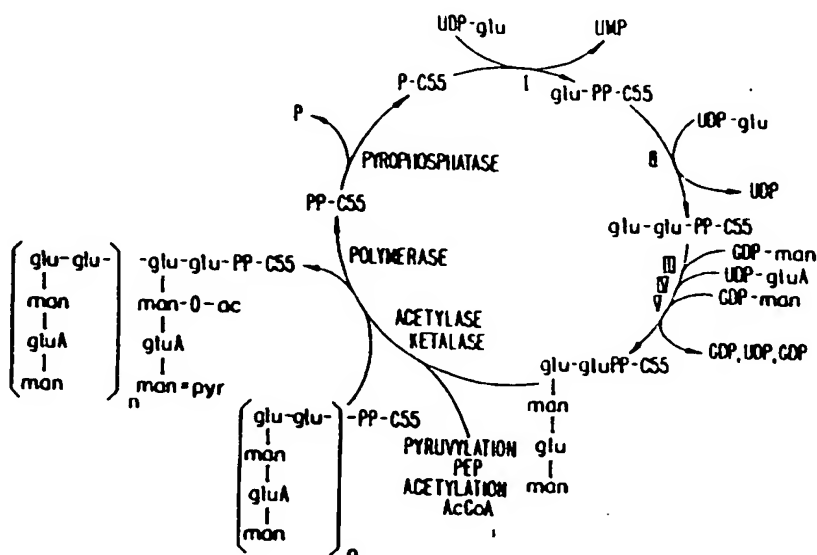
European Patent
Office

EUROPEAN SEARCH REPORT

Application Number

EP 96 11 9095

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.)
D,X	US 4 296 203 A (PFIZER INC.) * the whole document *	1-9	C12P19/06 C08B37/00 //C12R1:64
X	EP 0 066 961 A (KELCO BIOSPECIALTIES LIMITED) * the whole document *	9,11, 14-17	

TECHNICAL FIELDS
SEARCHED   (Int.Cl.)

C12P
C08B

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	5 February 1997	Mazet, J-F

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another
    document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or
    after the filing date
D : document cited in the application
L : document cited for other reasons
....
& : member of the same patent family, corresponding
    document

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mannose normally found on xanthan gum (Figure 4). A double
mutant plasmid, pKBm2delCla, was constructed which contains
an insertion mutation within gene gumI and the ClaI deletion
mutation within gene gumF (see Figure 5). The double mutant
plasmid pKBm2delCla was transferred into the X. campestris
deletion strain X1106 which contains only gum genes B and C
in its chromosome. Genes B and C are provided by the
chromosome since the mutant plasmid, derived from pRK290-HA3,
does not carry B or C but contains all the remaining gum
genes, D through M. The resulting strain, X1106(pKBm2delCla)
or X1419, was analyzed for polymer composition twice. Both
analyses failed to detect acetate in the polymer. This
result shows that Acetylase II cannot acetylate the internal
mannose of the polytetramer to any significant degree. In
this mutant strain, Acetylase II is active because gene gumG
is not mutated and the gene gumF mutation is the non-polar
ClaI deletion which has been shown above not to affect the
expression of gene gumG.

EXAMPLE 4

This example describes the repeating units that
comprise the polysaccharide family that can be produced by
genetic control of acetylation and pyruvylation of the
pentasaccharide repeating unit of xanthan gum. The
structures of these repeating units are shown in schematic
form in Figure 6.

(a)   Wild-type (X1396); Acetylase I$^+$, Acetylase
       II$^+$, Ketalase $^+$.

Normal xanthan is extensively acetylated at the inner
mannose residue and is frequently pyruvylated on the outer
mannose residue. Contrary to general belief, a significant
percentage (10-20) of the outer mannose residues of normal
xanthan are acetylated. Thus, normal xanthan repeating units
are heterogeneous with respect to modifications of the outer
mannose, containing either pyruvate or acetate.

(b) L^-(X1397); Acetylase I$^+$, Acetylase II$^+$, Ketalase$^-$.

This polymer contains no pyruvate and as a result is extensively acetylated at the outer mannose residue. The inner mannose residue is highly acetylated as in wild type.

(c) G^- (X1398); Acetylase I$^+$, Acetylase II$^-$, Ketalase$^+$.

This polymer is heavily acetylated on the inner mannose as in wild type, and the outer mannose is pyruvylated in the wild-type fashion. However, there is no acetylation of the outer mannose.

(d) G^-, L^- (X1399); Acetylase I$^+$, Acetylase II$^-$, Ketalase$^-$.

This polymer has the high level wild-type acetylation of the inner mannose, but the outer mannose is unmodified.

(e) F^- (X1400); Acetylase I$^-$, Acetylase II$^+$, Ketalase$^+$.

The inner mannose of this polymer is unmodified, while the outer mannose is modified as in wild-type. That is, the outer mannose in generally pyruvylated, but a significant fraction of the outer mannose residues are acetylated instead.

(f) F^-, L^- (X1401); Acetylase I$^-$, Acetylase II$^+$, Ketalase$^-$.

This polymer contains an unmodified inner mannose. The outer mannose is not pyruvylated but is heavily acetylated.

(g) F^-, G^- (X1402); Acetylase I$^-$, Acetylase II$^-$, Ketalase$^+$.

This polymer is not acetylated at either the inner or outer mannose residues. Pyruvylation of the outer mannose occurs normally as in wild-type.

(h) F^-, G^-, L^- (X1403); Acetylase I$^-$, Acetylase II$^-$, Ketalase $^-$.

This polymer contains no acetate or pyruvate. Neither the inner nor the outer mannose residues are modified.

EXAMPLE 5

This example describes the construction of a chromosomal deletion mutation defective in both acetylase genes gumF and gumG.

The variant xanthans described in Example 4 are produced by mutant strains of X. campestris in which the gum gene cluster has been deleted from the chromosome and is present in the cell on a recombinant plasmid. In some instances it might be desirable to have the gum gene cluster located in the X. campestris chromosome as this would eliminate the need for plasmid maintenance, and thus should improve strain stability. A chromosomal deletion mutation defective in genes gumF and gumG, which encode Acetylase I and II, respectively, was constructed as described below. This mutant produces non-acetylated xanthan gum.

Figure 7a shows a map of a 4.3 Kb BglII fragment of the gum gene cluster that contains the gumF and gumG genes. This BglII fragment is cloned into the BglII site of the plasmid vector pS1 to generate a recombinant plasmid termed pS1Bgl. The pS1 vector is a derivative of pRK290 in which the origin of vegetative, but not conjugal, DNA replication is replaced by the replication origin of pBR322. The pS1 replication origin does not function in X. campestris. Therefore, pS1 and its derivatives, such as pS1Bgl are "suicide" plasmids, i.e., they can be conjugally transferred into X. campestris but cannot replicate in X. campestris.

The three ClaI sites shown in Figure 7a were used in construction of the deletion mutation. Deletion of the ClaI fragment internal to the gumF gene was accomplished as described in Example 1 (by digesting pS1Bgl with ClaI and religating at low DNA concentration) to produce pS1BglΔCla shown in Figure 2b. In those experiments digestion at the third ClaI site within gene gumG was not observed, although the DNA sequence at this site is the ClaI recognition site, ATCGAT.

However, digestion by ClaI is known to be sensitive to methylation of the first adenine (A) residue of the recognition sequence. If that residue is methylated, the enzyme will not cleave the sequence. In E. coli, the sequence GATC is a substrate for the dam methylase which methylates the A residue of this tetranucleotide sequence. The ClaI recognition site in gene gumG is preceded by a G residue. Thus, in that sequence, GATCGAT, the underlined A is methylated because it occurs within the GATC tetranucleotide sequence; and therefore, the ClaI site is refractory to ClaI digestion.

In order to digest the ClaI site within gene gumG, it was necessary to produce unmethylated DNA by propagating pS1Bg1ΔCla in an E. coli dam strain which is defective for the relevant methylase activity. When unmethylated pS1Bg1ΔCla DNA was prepared, digestion at the gene gumG ClaI was readily observed. Using this plasmid DNA as a substrate, the second ClaI fragment was deleted by digesting pS1Bg1ΔCla with ClaI and religating at low DNA concentration to produce pS1Bg1ΔCla2 which is shown in Figure 7c. The resultant deletion of both ClaI fragments creates an in-frame fusion of the proximal portion of the gumF gene with the distal portion of the gumG gene. Therefore, transcription and translation through this region, and of distally located genes H through M (Figure 1), should proceed normally.

The plasmid pS1Bg1ΔCla2 including the deletion mutation was transferred into the X. campestris chromosome via gene replacement. This two-step recombination procedure is diagrammed in a general fashion in Figure 8. In general, in the first step, homologous recombination between the plasmid and the chromosome results in integration of the whole plasmid into the chromosome. This event is selected by selection for maintenance of an antibiotic resistance marker carried within the vector portion of the plasmid. In the second step, a subsequent recombination results in loss of the plasmid and retention of the constructed deletion mutation. This event is detected by the screening for loss

of the antibiotic-resistance determinant and Southern blot
hybridization analysis of chromosomal DNA structure.

In the present example, pS1BglΔCla2 was conjugally
transferred into wild type X. campestris strain X77 by
standard triparental matings and tetracycline-resistant
transconjugants were selected. These tetracycline-resistant
colonies were either light or dark yellow in appearance. DNA
was prepared from four of each (light and dark type),
digested with BamHI (pS1BglΔCla2 contains no BamHI sites),
separated by agarose gel electrophoresis and Southern blot
hybridization was performed. Probing with labeled gum gene
cluster DNA showed a wild type pattern in DNA from the light
yellow isolates. The hybridization pattern for DNAs from the
dark yellow isolates indicated that, in these isolates,
pS1BglΔCla2 had recombined into the chromosome.

Two of the bonafide pS1BglΔCla2 insertion isolates were
then grown in the absence of tetracycline to screen for the
subsequent recombination in the second step of the gene
replacement process. In these experiments, liquid cultures
are grown to saturation in the absence of tetracycline,
diluted 1:50 in fresh growth medium, and again grown to
saturation. In several experiments of this type, the number
of subculturing steps varied from 2 to 6.

Following the liquid subculturing steps, the cells were
diluted and plated, in the absence of tetracycline, to yield
isolated colonies. These colonies were tested for
tetracyclineresistance. In different experiments, the
frequency of tetracycline-sensitive colonies varied widely,
from ~.5% to >50%. The frequency of tetracycline-sensitive
isolates increased with the number of generations of growth
(i.e. subculture steps) in the absence of tetracycline.

Individual tetracycline-sensitive isolates were grown and
their chromosomal DNAs analyzed by Southern blot
hybridization to identify those isolates which contained the
deletion mutation in the chromosome and had lost the plasmid
sequences as a result of homologous recombination. One such
isolate, termed X1910, was characterized for polysaccharide

polymer production _in vivo_ by the procedures detailed in Example 2.

The composition of the polysaccharide polymer produced by X1910 was found to be indistinguishable from wild type xanthan with respect to glucose, mannose, glucuronic acid and pyruvate; whereas, in contrast, no acetate was detected in the gum made by X1910. Thus this mutant produces non-acetylated xanthan having a composition equivalent to that produced by X1402 (Example 4), but does so as a result of the chromosomal deletion mutation. No recombinant plasmid or any foreign DNA is present in this strain.

The general approach and strategy described above could readily be applied to create analogous chromosomal deletion mutants that would produce any of the variant xanthans described in this application or in Vanderslice and Shannon U.S. Patent No. 4,713,449.

EXAMPLE 6

This example shows the methods of mutagenesis and screening employed to generate X. campestris mutant strains having defects in Acetylase or Ketalase activity.

The genes encoding the enzymes of xanthan gum biosynthesis have been shown to comprise a set of clustered genes on the X. campestris chromosome. This "gum gene cluster" has been described in detail by Capage _et al_. Segments of gum gene DNA have been cloned on plasmid vectors such as pMW79 as detailed in Capage _et al_.

Regionally-directed mutagenesis was performed upon subcloned portions of the gum DNA carried in plasmid pMW79. These cloned DNA segments were mutagenized _in vivo_ with transposons and _in vitro_, by using recombinant DNA technology to generate insertion, deletion, and substitution mutations within the cloned X. campestris DNA. In order to study the phenotypes conferred by these mutations, the plasmids carrying the mutations were transferred back into X. campestris and subsequently recombinants were identified in which the plasmid-borne, mutated gene had been inserted in the chromosome via homologous recombination. The

tetracycline resistance encoded by Tn10 affords a convenient selective system for movement of mutations from a plasmid into the chromosome.

One such mutant strain (X1006) carried a Tn10 insertion that was found to cause inactivation of the Acetylase activity. This mutant strain was characterized as described in ExampleS 7 and 8, and found to produce a polysaccharide that was non-acetylated. A second mutant strain was constructed by the in vitro insertion of a fragment of DNA containing the tetracycline resistance gene of Tn10 into a restriction site within the gum gene cluster. This mutant strain (X921) was found to be defective in the Ketalase activity. As found by the methods of Examples 7 and 8, this mutant produced xanthan that was non-pyruvylated.

A third mutant strain (X934) was also found that greatly reduces the ketalase activity. This mutant strain produces xanthan gum that has a very low level of pyruvylation: 1-5% of the level of pyruvylation found in normal xanthan.

The mutant strain X934 was found as described below. In preliminary experiments designed to study recombination between plasmid-borne X. campestris DNA and the X. campestris PstI fragment cloned in plasmid RSF1010. This insertion of Tn10 causes the Gum- defect in the mutant strain X655 as described by Capage et al. and Vanderslice et al. The experiment was to mobilize PTX655 with plasmid pRK2013 and transfer it from E. coli into X. campestris by selecting for transfer of the tetracycline resistance encoded by Tn10. The initial results of this mating were anomalous and suggested that Tn10 did not express tetracycline resistance efficiently in X. campestris when carried on the plasmid, but that the drug resistance was more efficiently expressed when Tn10 was carried in the chromosome of X. campestris. This phenomenon has also been described for Tn10 in E. coli. There, it has been shown that strains carrying one copy of Tn10 inserted in the chromosome are resistant to significantly higher concentrations of tetracycline than are strains carrying Tn10 on a multicopy plasmid. The selection of Tet^r X. campestris

out of the above mating resulted in a high frequency (0.5 per
recipient) of progeny which grew very poorly (i.e., only
small, watery colonies) on tetracycline. After prolonged
incubation, a large fraction of the colonies (25%) produced
sectors of more vigorously growing cells. More than 50% of
these sectors appeared to be Gum- in morphology. These
probably result from recombination between the plasmid-borne
DNA containing the Tn10 insertion and the chromosomal wild
type DNA. When the Tn10 is recombined into the chromosomal,
high-level Tet^r is obtained and the vigorously growing sector
is observed. When these Gum-, Tet$_r$ sectors were picked and
restreaked on tetracycline, they grew well and displayed a
characteristic Gum- morphology.

Gum+, Tet^r isolates were also characterized. Some of
these strains (in particular X934) were found to contain the
entire plasmid pTX655 inserted into the chromosome of X.
campestris via homologous recombination. The chromosomal
structure of the X934 strain was determined by Southern blot
hybridization of the chromosomal DNA which shows that the
plasmid sequences exist in a chromosomally integrated form.

EXAMPLE 7

This example shows how the altered polysaccharides of the
present invention can be prepared in vitro. For instance, it
shows how non-acetylated and/or non-pyruvylated xanthan gum
was prepared in vitro.

Preparation of Lysates

Xanthomonas campestris B1459 S4-L or S4-L mutants
described in Examples 6 and 10 were grown in YM (yeast-malt
medium) supplemented with 2% (w/v) glucose as described by
Jeanes, A. et al. in U.S. Department of Agriculture, ARS-NC-
51, pp. 14 (1976), specifically incorporated hereby by
reference. Cultures were grown to late log phase at 30°C.
The cells were harvested by centrifugation and washed with
cold Tris-HCl, 70mM, pH 8.2 with 10mM EDTA and were freeze-
thawed three times by a procedure similar to Garcia, R.C. et
al. described in European Journal of Biochemistry 43:93-105
(1974), specifically incorporated hereby by reference. This

rate of 8 s^{-1} in a concentric cylinder viscometer on solutions of 1000 ppm active polymer solids in 5000 ppm NaCl brine.

EXAMPLE 13

This example demonstrates the advantages of a non-acetylated xanthan produced by a genetically modified Xanthomonas campestris for use as a viscosifying agent for aqueous solutions.

Figure 10 compares the viscosities of chemically deacetylated commercial xanthan and its parent compound with those of a non-acetylated xanthan polysaccharide made from a genetically manipulated Xanthomonas campestris (strain X1006) and of the xanthan gum made from the wild-type X. campestris parent (strain X237). The viscosities were obtained at a shear rate of 8 s^{-1} for 1000 ppm polymer concentration in 50,000 ppm NaCl brine. Viscosities were measured over the temperature range of 25° to about 80°C.

Figure 10 demonstrates that chemical deacetylation of xanthan results in a loss of viscosifying power over the entire temperature range. However, elimination of acetylation by genetic means results in substantially increased viscosity compared to the wild-type xanthan gum. Thus, non-acetylated xanthan gum produced by a mutant strain of X. campestris is an improved polysaccharide compared to the xanthan itself and compared to deactetylated xanthan gum produced by chemical methods.

EXAMPLE 14

This example describes the methods used to construct a double mutant strain of X. campestris that produces non-acetylated, non-pyruvylated xanthan.

Capage et al. have described the cloning of a gene cluster from X. campestris that contains genes that direct the biosynthesis of xanthan gum. They also described the isolation of chromosomal deletion mutations in X. campestris that eliminate all or varying portions of this gene cluster. One such deletion mutant, strain X1231, lacks all of the X. campestris DNA that is carried on the recombinant plasmid
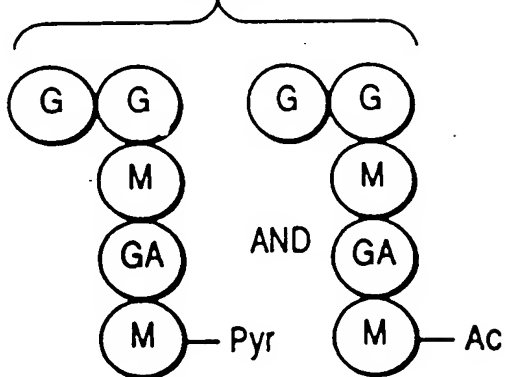
FIG. 6a
WILD TYPE (x1396)
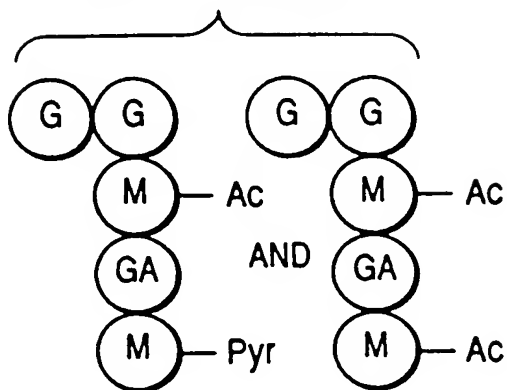
FIG. 6b
L⁻(x1397)
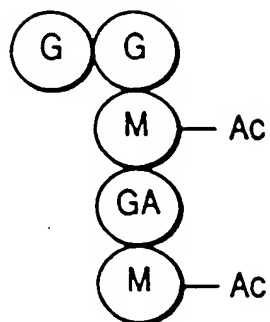
FIG. 6c
G⁻(x1398)
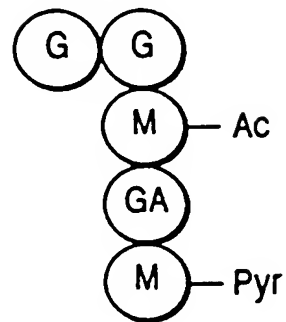
FIG. 6e
F⁻(x1400)

FIG. 6d
G⁻L⁻(x1399)
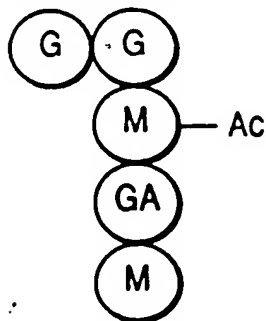
FIG. 6f
F⁻L⁻(x1401)
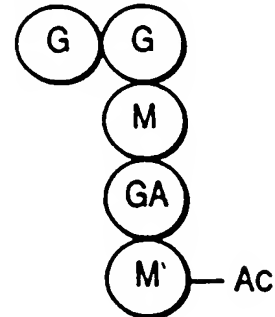
FIG. 6g
G⁻F⁻(x1402)

FIG. 6h
G⁻F⁻L⁻(x1403)

SUBSTITUTE SHEET

FIG. 10